CI. THE DUAL NATURE OF WATER-SOLUBLE VITAMIN B. II.

THE EFFECT UPON YOUNG RATS OF VITAMIN B_2 DEFICIENCY AND A METHOD FOR THE BIOLOGICAL ASSAY OF VITAMIN B_2.

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In a previous communication [Chick and Roscoe, 1927], experiments were described which confirmed the conclusion of Goldberger and his colleagues [1925, 1926] that the water-soluble B vitamin as defined by the discoverers, McCollum and Davis [1915, 1, 2], had two components[1].

(1) Vitamin B_1 or the antineuritic, less heat-stable vitamin discovered by Eijkman in 1897, deficiency of which in diet leads to polyneuritis and death in birds, and to death, with or without paralysis, in rats and other mammals.

(2) Vitamin B_2, a more heat-stable vitamin, often accompanying vitamin B_1 in natural foodstuffs, in the absence of which the animal fails to grow and, in the case of rats, suffers from dermatitis.

Deficiency of vitamin B_1 is usually held to be the cause of beriberi in man, and, according to Goldberger and his colleagues, deficiency of vitamin B_2 is the cause of human pellagra.

When young rats are placed upon an experimental diet complete in all other respects, they show no growth if *either* of these two vitamins is lacking. In absence of vitamin B_1, death usually occurs in 3–4 weeks; in absence of B_2, the rats survive much longer. There is, therefore, no justification for calling vitamin B_2 a "growth factor" in contradistinction to vitamin B_1, as has been done by some writers.

That the water-soluble B vitamin and the antineuritic vitamin were not identical, as was supposed by McCollum and Kennedy [1916] and others, has long been suspected and a résumé of the literature on the subject was given in our previous paper [Chick and Roscoe, 1927]. Since the work of Goldberger and his colleagues was published, however, many papers besides our own have appeared which bear upon this point. In some, the separate action of vitamins B_1 and B_2 is clearly demonstrated, in others, it can be inferred.

[1] The nomenclature here used is in accordance with that provisionally adopted by the Biochemical Society.

Smith and Hendrick [1926] found that a synthetic diet devoid of water-soluble B vitamins was not rendered adequate to maintain growth in rats by adding Seidell's antineuritic concentrate (picrate) prepared from yeast, but required to be supplemented by an additional dietary factor. This second factor was also contained in yeast, and was able to withstand heating in the autoclave at 15 lbs. pressure for 6 hours.

Hauge and Carrick [1926], from observations on the growth and development of baby chicks, concluded that yeast contained a water-soluble vitamin necessary for growth which was separate from the antineuritic factor.

Salmon [1927] found that the seeds of the velvet bean were more potent than the leaves in preventing polyneuritis in pigeons fed on polished rice, but that the reverse was true for maintenance of growth in young rats when one or other of these two products was used to supplement experimental diets devoid of water-soluble B vitamins. Salmon succeeded in making a partial separation of the two water-soluble vitamins from the leaves of the velvet bean by adsorbing the antineuritic vitamin on fuller's earth, but in order to maintain growth in rats this antineuritic solid required to be supplemented with the filtrate yielded after treatment with the earth. In a later paper Salmon, Guerrant and Hays [1928] reported a more complete separation of these two principles to which they gave the names vitamin P–P, as suggested by Goldberger (= B_2) and B–P (= B_1). They found (1) that vitamin B_1 was adsorbed without appreciable admixture of vitamin B_2 if the extract made from the leaves were treated with a very small quantity of fuller's earth (10 g. per kg. air-dried leaves), and (2) that vitamin B_2 was precipitated with very small admixture of vitamin B_1, if alcohol to 82·7 % (by weight) were added to the leaf extract, after the latter had been previously treated several times with fuller's earth to remove vitamin B_1.

Williams and Waterman [1927], also using fuller's earth, removed a substance from an aqueous extract of yeast which was antineuritic but unable to maintain growth of rats on diets devoid of water-soluble B unless supplemented, for example, by yeast which had been heated for 6 hours at 125°.

Sherman and Axtmayer [1927] found that a supplementary relationship existed between whole wheat and autoclaved yeast and between whole wheat and dried skimmed milk when these were used to supply "vitamin B" to an otherwise complete diet. From these results the authors deduce the existence of two separate dietary factors in "vitamin B," both necessary for growth, for which they propose the names vitamin F (antineuritic) and vitamin G (heat stable).

In a series of recent papers Palmer and Kennedy [1927, 1, 2; 1928], have described the difficulties encountered in designing a "synthetic" diet, prepared from highly purified foodstuffs, which should be entirely satisfactory for growth and reproduction of rats. "Vitamin B" was supplied by an alcoholic extract of wheat embryo and highly purified caseinogen was the source of protein. The nutritive value was much improved when the diet was supple-

mented by autoclaved yeast or when commercial caseinogen was substituted for the highly purified product (see below, p. 793). It seems probable, though not proved, that vitamin B_2 is the missing constituent in their apparently complete "ration 5." This conclusion agrees with the results of recent investigations by Evans and Burr [1928] on the difference in growth of rats maintained on rations containing, respectively, purified caseinogen and sugar and the commercial products. They conclude that vitamin B_2 is present in the latter but not in the former. They also find that "Tikitiki," the dilute alcoholic extract of white rice polishings distributed to the natives by the Philippine Bureau of Science, possesses the antineuritic vitamin while "almost entirely lacking the growth-promoting vitamin B."

The technique which enabled us to demonstrate the separate effect on nutrition of these two vitamins was the use of the concentrated antineuritic vitamin prepared from yeast by the method of Peters [Peters, 1924; Kinnersley and Peters, 1925]. This product contains vitamin B_1 without any admixture of vitamin B_2 [Chick and Roscoe, 1927]. As a source of vitamin B_2, free from B_1, we used whole yeast autoclaved for 5 hours at 120°. This degree of heating destroys all the antineuritic vitamin contained in yeast, but only about one-half of the vitamin B_2 originally present.

Since our earlier paper was written some improvements in animal technique have been devised which have enabled us to make a more accurate study of the effect of vitamin B_2 deficiency on young growing rats. Incidentally, a method has been evolved for evaluation of vitamin B_2 which appears to possess a reasonable degree of accuracy for a biological method.

Improvements in animal technique required for study of vitamin B_2.

In our earlier experiments [Chick and Roscoe, 1927] we found that young rats on diet L[1] (devoid of water-soluble vitamins) and receiving vitamin B_1, in the form of a small daily ration of Peters's antineuritic concentrate, failed to grow and within 7–10 weeks developed the dermatitis and skin lesions described by Goldberger and Lillie [1926]. In subsequent experiments, however, skin symptoms were absent, although the animals were maintained for long periods on the diet and no significant increase in weight occurred. Other workers, *e.g.* Macy, Outhouse, Long and Graham [1927], have had the same experience.

By analogy with observations made upon other vitamins, it seemed possible that the irregularity might be due to variations in the reserves of vitamin B_2

[1] Diet L, devoid of water-soluble B vitamins:

Purified caseinogen	100 g.
Rice starch	300 g.
Cotton seed oil	75 g.
Salt mixture (McCollum's No. 185)	25 g.
Water	500 cc.

Cod-liver oil given daily by hand 0·05–0·1 g., according to the size of the rat.
The diet is heated, before use, for 3 hours in steam at 100°, in order to prevent the possibility of "refection" in the experimental animals [see Roscoe, 1927].

held by the animals. Although the young rat is incapable of storing any considerable quantity of vitamin B_1, the long period of survival when deprived of vitamin B_2 suggested a capacity for its storage.

Attempts were, therefore, made to reduce the supply of water-soluble B vitamins during lactation by limiting the mother's diet to rice, polenta (maize endosperm), white bread and milk, marmite being omitted. In case of one litter thus bred, 2 out of 3 receiving the "$-B_2$" diet developed the typical skin appearances; of these, one (No. 212) died, but the other (No. 213) showed some degree of spontaneous recovery. In the case of another litter, two rats (Nos. 172 and 178) survived more than 20 weeks without developing any skin lesions. These results showed no improvement in consistency and it was necessary to look elsewhere for the causes of the irregularity.

We next considered the possibility that our basal diet might not be sufficiently free from vitamin B_2. We had hitherto used caseinogen prepared commercially from skim milk by precipitation with acid, washing with acidulated water, drying and prolonged heating at 120°. The caseinogen was not extracted with alcohol, so that, while the heating to which it was subjected might have destroyed vitamin B_1, vitamin B_2 might still be present, and our irregular results might be due to the use of batches of caseinogen containing different amounts of B_2.

We therefore purified caseinogen as follows. "Light white soluble" caseinogen was dissolved to form a 5 % solution, reprecipitated with acetic acid and washed by decantation with 0·05 % acetic acid, the supernatant fluid being changed twice a day for a fortnight. The caseinogen was then extracted with dilute acidified alcohol (50–70 % strength (by volume), containing about 0·03 % acetic acid) for 96 hours in a large Soxhlet apparatus, and afterwards washed with 93 % alcohol, dried in an electric oven at a low temperature, ground in a mill and finally roasted for 3 days at 120°.

The behaviour of young rats 40–50 g. in weight receiving the "$-B_2$" diet prepared with this highly purified caseinogen was compared with that of others from the same litter receiving a similar diet made with the caseinogen previously used. After 12 weeks the difference between the two sets of rats was striking. Two rats receiving the commercially purified caseinogen had poor coats and had shown no significant increase in weight (weight 52 g.) but they were in fair condition, they showed no skin symptoms, and their eyes remained normal (see rats 218 and 222, Fig. 1). The weight of 7 rats receiving the specially purified caseinogen ranged from 39 to 47 g., 5 showed typical skin symptoms, all had sunken eyes and inflamed eyelids and their general condition was miserable (see rats 217, 220 and 221, Fig. 1).

It is evident, therefore, that vitamin B_2 is present in precipitated caseinogen, and can be removed by thorough extraction with acid water and acid alcohol. This is a point of some importance and, in part at least, may account for the high nutritive value possessed by caseinogen as a protein. It probably explains the improvement observed by Palmer and Kennedy [1927, 2] in the nutri-

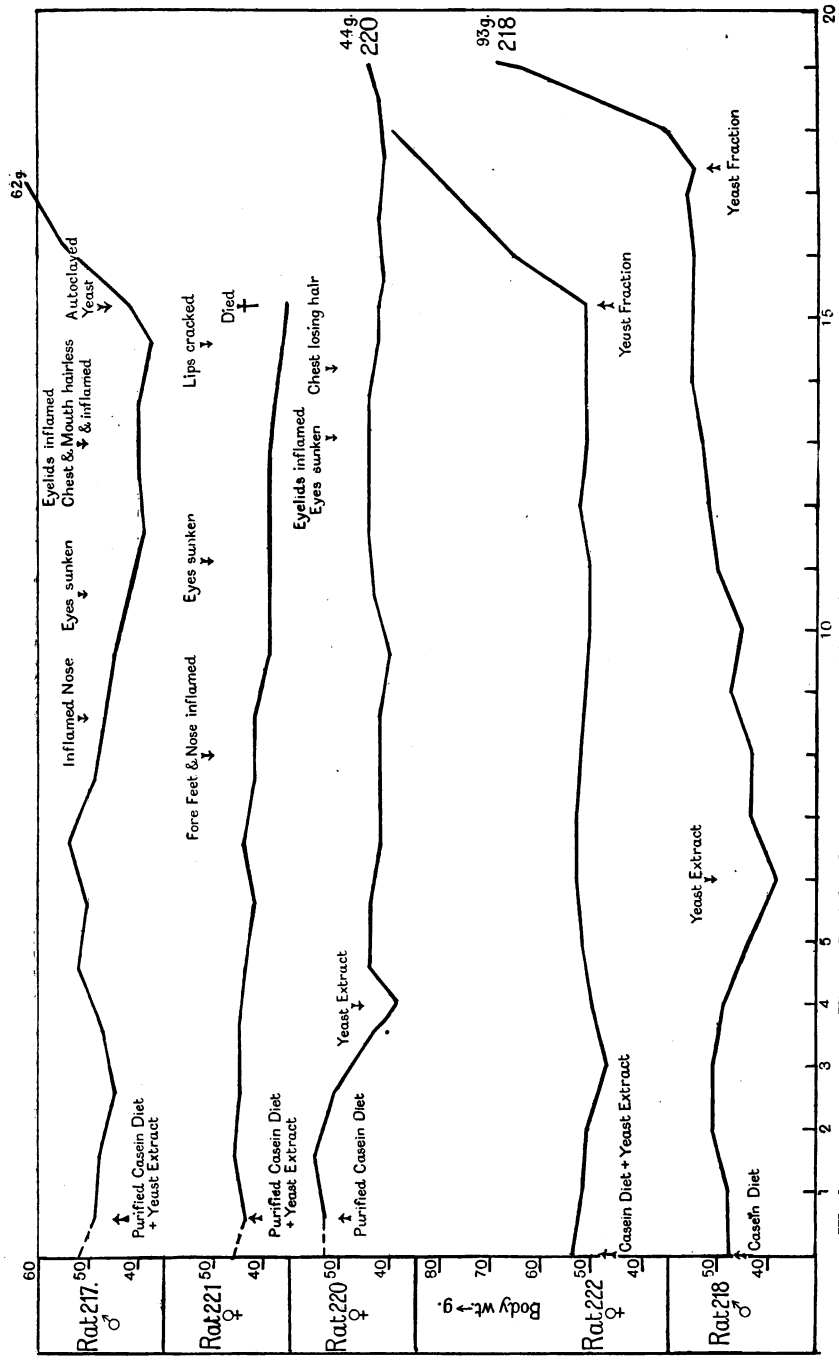

Fig. 1. Weight charts of rats on diets devoid of water-soluble B vitamins.
Yeast extract contains B_1. Yeast fraction contains B_2.

tional value of their "synthetic" ration for rats, when a commercial preparation of caseinogen was substituted for their highly purified product. The disappointing results obtained by Goldberger and Tanner [1925] with highly purified caseinogen, as compared with milk and milk products, when these were added to the diet of inmates in Institutions where pellagra was endemic, may possibly have a similar explanation.

Symptoms shown by young rats on a diet deprived of vitamin B_2.

Young rats can often be maintained for 3 months and longer upon a diet deprived of vitamin B_2. During this period they will show no significant increase or loss in weight (see Fig. 1). They do not, however, appear to be ill or distressed, they have a fair appetite and remain active and interested in their environment. The skin symptoms, which are symmetrically placed on the animal, gradually develop at any time after the 5th or 6th week, but do not usually become severe until several weeks later. The most constant symptoms in our experience are the following. (1) Dermatitis and loss of hair from the eyelids,which may become stuck together; if the eyelids are loosened by bathing with warm water, the eyes, though much sunken, appear to be healthy. (2) Front paws stained with blood, caused by rubbing the inflamed margins of the nostrils; wetting of the lower portion of the abdomen with blood stained urine. (3) Dermatitis and loss of fur on head, round the nose and mouth and on the abdomen. Inflammation of the skin at the tips of the ears and a curious and very characteristic oedematous dermatitis of the digits of the paws, which become bright red in colour, were constant early symptoms in our earlier experiments, but at present are encountered later and much less frequently.

The irregularity in the occurrence of the various skin symptoms and the occasional instances of animals deprived of vitamin B_2 remaining stunted in growth but exhibiting no special skin lesions, suggest that we may possibly be dealing with more than one kind of dietary deficiency. There has certainly

Fig. 1. Weight charts of 5 young rats from the same litter on basal diets devoid of water-soluble B vitamins. Vitamin B_1 given as Peters's concentrated antineuritic extract from yeast (daily dose $\equiv$0·6 g. dry yeast). Rats 217, 220, 221 and rats 218, 222, received diets prepared from the more and less highly purified caseinogen respectively.

Rat 217 (initial weight, 52 g.) received vitamin B_1 throughout, developed typical skin lesions after 9 weeks; at 15 weeks (weight 39 g.) received vitamin B_2 as 0·4 g. daily of yeast autoclaved for 5 hours at 120°.

Rat 221 (initial weight, 46 g.) treated similarly to rat 217, developed skin lesions after 8 weeks, remained untreated and died after 15 weeks of vitamin B_2 deficiency (weight 35 g.).

Rat 220 (initial weight 53 g.) at first deprived of both B vitamins, showed collapse (weight 38 g.) due to lack of vitamin B_1 at $4\frac{1}{2}$ weeks, was restored by administration of vitamin B_1, developed skin lesions after the 13th week (weight after 19 weeks, 44 g.).

Rat 222 (initial weight 54 g.) received vitamin B_1 throughout; at 15 weeks had developed no skin symptoms (weight 51 g.); administration of vitamin B_2 in the form of a yeast fraction, caused increase in weight of 39 g. in 3 weeks.

Rat 218 (initial weight 48 g.) at first deprived of both B vitamins, showed collapse (weight 38 g.) due to lack of vitamin B_1 at 6 weeks, restored by administration of vitamin B_1, showed no skin symptoms in 18 weeks (weight 54 g.); administration of vitamin B_2, in form of a yeast fraction, caused immediate increase in weight (46 g. in 2 weeks).

been greater constancy in the development of symptoms since we have taken steps to free the caseinogen in the basal diet from possible contamination with water- or alcohol-soluble vitamins, but, on the other hand, the typical dermatitis of the paws and ears, for example, which is now so infrequent, was previously often observed in rats upon diets prepared with less pure caseinogen and receiving a small, though insufficient, supply of vitamin B_2 in the form of autoclaved yeast [see Chick and Roscoe, 1927, Table II].

Our experience also differs in some respects from that of Findlay [1928] who records "moderate growth...for from 4 to 6 weeks" in rats deprived of vitamin B_2, followed by "slight but gradual loss of weight" with development of skin lesions. We have not observed the change in temper of the animals, those previously tame and docile becoming irritable and liable to bite, described by him, nor have we observed any great loss in appetite. In our experience, the intake of food remains rather high (about 4–6 g. daily dry weight) seeing that the body weight of the animals remains steady at about 40–50 g.

Recovery on addition of vitamin B_2 to the diet.

If the condition of the rats after many weeks on the "$-B_2$" diet is not too bad, they respond promptly to administration of vitamin B_2 and increase in weight begins immediately, often within 24 hours. If the supply of vitamin B_2 is abundant the growth in weight may be far in excess of the normal for a time and we have frequently observed gains of 20–30 g. a week for a short period (see Table I and Curves of rat 218, Fig. 1). The sunken eyes are usually the next to show improvement and soon begin to protrude. Desquamation of the affected skin also takes place, after which the fur begins to grow and the animal gains a normal appearance within 2–4 weeks, according to the severity of the previous symptoms. It remains for some time undersized for its age.

Method for evaluation of vitamin B_2.

If the vitamin B_2 is supplied after only a few weeks of deprivation and before the animal's condition is too bad, the response in growth is at first roughly in proportion to the amount of vitamin B_2 administered. It is well, however, not to leave the animal too long on the deficient diet (see rat 251, Table I) and there is no need to await the development of specific symptoms.

Young rats are placed upon the basal diet, deprived of B vitamins, when about 4 weeks old and 40–50 g. in body weight. They are left together in a large cage for about 10 days. After this time, they are placed in separate cages, because of their tendency to pull out and eat the fur of their companions. The cages have floors of wide-meshed ($\frac{1}{3}$-inch) zinc gauze, raised about 2 inches above trays filled with peat moss litter. This precaution is necessary in order to hinder the consumption of the faeces, which is a common practice of rats on diets deprived of B vitamins, and tends to obviate the effect of the dietary deficiency [Steenbock, Sell and Nelson, 1923].

Table I. *Titration of vitamin* B_2 *by increase of body weight in young rats, which have ceased to grow on "* $-B_2$*" diet, i.e. diet* L, *devoid of* B *vitamins, to which vitamin* B_1 *is added as Kinnersley and Peters's concentrate from yeast.*

Material tested	Daily dose expressed as the equivalent of dry yeast g.	Rat No.	Body weight when dose was started g.	Weekly growth increments while receiving dose g.	Mean for rats receiving a given dose g.	Time previously maintained on "$-B_2$" diet Weeks
Fraction 5 I, extract of Yeast V in 0·01 % acetic acid evaporated to small bulk	1·2	142	116	36, 32	26	14
	1·2	140	101	24, 12		14
	0·9	243	45	26, 22	24	3
Fraction 5 II, prepared as 5 I, from Yeast V and VII	0·5	254	41	21	21	2
	0·5	255	41	22		2
	0·5	256	43	19		2
	0·25	254	71	14, 11	11	2 + 2 on above test + 2 deprived until weight was again constant
	0·25	255	65	14, 12		
	0·25	256	66	8, 11		
Fraction 5 III, prepared as 5 I, from Yeast VIII	1·0	251	45	24, 28	26	15 + 1 receiving the equivalent of 0·5 g. yeast with no response
	0·5	251	43	2	—	15
	0·5	268	52	20, 15·5	18	6
	0·25	272	53	10·5, 13·5	11	7
	0·25	279	38	16, 17		4
	0·25	280	39	11, 11		2
	0·25	273	56	11, 9, 5, 9·5		2 + 2 receiving the equivalent of 0·12 g. yeast
	0·12	273	46	7, 3	7	2
	0·12	290	37	9, 12, 3		2
Dried Yeast	0·4	—	—	—	23	See Chick and Roscoe [1927], Table III
	0·2	—	—	—	11	

The daily dose of Peters's antineuritic concentrate is omitted at first, for reasons of economy, but after about 2 weeks, it is given. A daily dose of 0·1 cc. (equivalent to 0·6 g. of the original yeast, dry weight) provides an excess of vitamin B_1. It is necessary to remove all traces of lead and mercury from the preparation, seeing that it is to be fed regularly over long periods. This is conveniently done after the precipitation with Hopkins's reagent (acid mercuric sulphate) and before adsorption on norite charcoal. If precipitation with baryta is inserted after treatment with lead acetate, as recommended recently by Kinnersley and Peters [1927], the separation of the metallic sulphides is much facilitated.

The weight of the rat sometimes showed a temporary increase of a few grams after this inclusion of vitamin B_1 in the diet. After 3–4 weeks from the beginning of the experimental feeding, however, the young rat is prepared for testing the content of vitamin B_2 in any material which may be then administered. The minimum dose which gives an average weekly increase of 10–12 g. provides a convenient standard for comparison. It is not necessary

for the material tested to be freed from vitamin B_1, of which an excess is already present in the rat's diet.

Table I shows the results of titrating the vitamin B_2 content of a yeast extract made with 0·01 % acetic acid, the first filtrate obtained in the Kinnersley and Peters process for preparing an antineuritic concentrate from yeast. The tests were made on extracts from three different samples of yeast and show concordant results as long as the animals used have not been longer than 6–7 weeks on the deficient diet (see rat 251). For example, rat 268, which had been 6 weeks on the diet devoid of vitamin B_2, responded to a daily dose of Fraction 5 III, equivalent to 0·5 g. dry yeast, by an average increase in weight of 18 g. a week, whereas rat 251, after 15 weeks on the deficient diet, made no significant response to the same dose. This animal, however, grew 26 g. a week when it received the equivalent of 1 g. dry yeast. From the results obtained with rat 273 when testing Fraction 5 III, it appears justifiable to use the same rat successively for assay of two different doses of vitamin B_2, if the smaller dose is given first. If a larger is given first, an interval of 2 weeks without any dose should intervene before the smaller one is tested (see results of tests made with rats 254, 255 and 256 on Fraction 5 II).

The results obtained with Fractions 5 I, 5 II and 5 III, compared with those obtained with dried yeast, show that little loss of vitamin B_2 has taken place during the extraction.

Summary.

1. Additional evidence is given proving the existence of the two separate vitamins B_1 and B_2 in the "water-soluble B" vitamin complex (for definitions see p. 790).

2. An improved animal technique is described for the study of vitamin B_2, the most important modification being the use of highly purified caseinogen as source of protein in the basal diet. The antineuritic concentrate prepared by the method of Kinnersley and Peters is used as source of vitamin B_1.

3. A description is given of the effects observed in growing rats when they are fed on diets deficient only in vitamin B_2.

4. A method is described for assay of vitamin B_2.

5. It is suggested that the high value of caseinogen as a protein may be partly due to contamination with vitamin B_2 and that the accepted nutritional value for this and other proteins may need revision.

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